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A simple and efficient method for generation of induced pluripotent stem cells using piggyBac transposition of doxycycline-inducible factors and an EOS reporter system

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Abstract

PiggyBac (PB) transposition of reprogramming factors (Oct3/4 (O), Sox2 (S), Klf4 (K) and c-Myc) is a safe, non-viral method for generating induced pluripotent stem cells (iPSCs). However, compared to retroviral methods, the reprogramming efficiency of the PB-mediated methods is relatively low. In this study, we describe a simple and efficient system for generating high-quality iPSCs by a single transfection of multiple plasmids that does not require the use of a virus, special instruments or skilled techniques. To improve reprogramming efficiency, we modified the components of the polycistronic 2A vectors used in this study and also investigated the combination of another reprogramming-related factor (L-Myc). By simultaneous transposition of multiple PB vectors containing an EOS (Early Transposon promoter and Oct3/4 and Sox2 enhancers) reporter and modified polycistronic doxycycline (Dox)-inducible factors, we reprogrammed mouse somatic cells with an efficiency higher than is usually obtained with retroviral methods and we established some iPSC lines that contributed highly to chimeras. By using the Dox-inducible system, we also showed that the appropriate elimination of exogenous-factor expression at appropriate time accelerated the induction of *Oct3/4* when a combination of OKS and c-Myc vectors were used.

1. Introduction

Takahashi and Yamanaka (Takahashi & Yamanaka 2006) previously showed that four defined transcription factors (reprogramming factors; Oct3/4, Sox2, Klf4 and c-Myc) can reprogram somatic cells into induced pluripotent stem cells (iPSCs). iPSCs have great potential in human regenerative medicine. However, preclinical studies using large animals are necessary before iPSCs can be applied to humans. Although disease models in large animals were expected to be good models for preclinical studies, iPSC lines that can maintain pluripotency without the support of exogenous factors and that can contribute to germ line chimera are not yet available for these species. The goal of this study was to establish a system for screening culture conditions that can be used to generate iPSCs in various mammalian species. For this purpose, we chose a Doxycycline (Dox)-inducible system (Brambrink *et al.* 2008; Stadtfeld *et al.* 2008) and EOS-reporter system (Hotta *et al.* 2009) for generating iPSCs.

The Dox-inducible system can control the expression of reprogramming factors with or without Dox. Therefore, this system could be useful for examining whether the reprogrammed cells can maintain pluripotency without the support of exogenous factors. For example, “pre-” iPSCs that were maintained their pluripotency by the exogenous reprogramming factors could be generated by the Dox addition and could be passaged in different culture conditions containing various combinations of growth factors, serum replacements, small-molecule compounds, peptides, feeder layers and extracellular matrix. After the Dox withdrawal, the dependency of these pre-iPSCs to exogenous factors and an optimal culture condition for iPSCs could be analyzed.

In mice, iPSC colonies can be selected by the expression of reporter genes that are inserted into pluripotent genes such as *Oct3/4* or *Nanog* by gene targeting although the procedure is rather complicated (Maherali *et al.* 2007; Okita *et al.* 2007; Wernig *et al.* 2007). For most mammalian species other than mice, however, no ESC lines or iPSC

lines leading to the production of transgenic animals have been established. Therefore, the use of large genomic fragments such as bacterial artificial chromosome (BAC) is difficult and the production of transgenic animals having a pluripotent reporter is time consuming. The EOS contains conserved regions that act as enhancers of reporter genes and is functional in mouse and human ES/iPSCs as a pluripotent reporter (Hotta *et al.* 2009). Therefore, the EOS reporter system may be useful for selecting pluripotent-stem-cell colonies in various mammalian species as well as for generating mouse iPSCs from genetically unmodified cells.

To simultaneously introduce the Dox-inducible reprogramming factors and the EOS reporter into cells, we used piggyBac (PB) transposition. The PB transposon system (Kaji *et al.* 2009; Woltjen *et al.* 2009; Yusa *et al.* 2009) is a vector system for generating iPSCs, in which integrated constructs including reprogramming factors can be removed by reexpression of transposase (PBase). However, in our preliminary experiments, PB transposition was not as effective at generating iPSCs in mice as the usual method that uses retroviral vectors. To improve the efficiency of iPSC generation by using PB transposition, we focused on the order of introducing genes in the polycistronic 2A vectors used in this study. Okita *et al.* (Okita *et al.* 2008) reported that the order of genes in polycistronic retroviral vectors, Oct3/4, Klf4, and then Sox2 (OKS), can affect the efficiency of reprogramming and generation of iPSCs. In addition, replacing one of the four factors (c-Myc) with L-Myc was found to result in the generation of a large number of high quality iPSCs (Nakagawa *et al.* 2010).

In this experiment, we constructed PB vectors with a Dox-inducible cassette consisting of OKS, c-Myc, L-Myc or OKS and L-Myc (OKSLM) and compared the reprogramming abilities of these PB vectors on mouse embryonic fibroblasts (MEFs) with the reprogramming ability of a previously reported polycistronic vector containing c-Myc, Klf4, Oct3/4 and Sox2 (MKOS). We showed that the Dox-inducible PB vector

1 could efficiently reprogram mouse somatic cells compared to retroviral methods and
2 that appropriate shut-off of exogenous genes by the withdrawal of Dox could be
3 accelerated the endogenous *Oct3/4* expression.

4

5

6

[Insert Fig. 1.]

2. Results

2.1. Improvement of reprogramming efficiency.

Fourteen days after the Dox addition, GOF18 MEFs that were transfected with Dox-inducible MKOS, OKS, OKS+cM, OKS+LM or OKSLM vectors formed many mouse ESC-like colonies. Cells transfected with OKS+cM produced far more colonies than cells transfected with other combinations (Fig. 2). Most of these colonies, however, were negative for *Oct3/4*-GFP and the positive rate was only $0.73 \pm 0.23\%$. By contrast, most of the colonies from cells that were transfected with MKOS ($90.61 \pm 6.31\%$), OKS+LM ($57.07 \pm 5.53\%$) or OKSLM ($66.39 \pm 15.16\%$) were positive for *Oct3/4*-GFP (Fig. 2).

[Insert Fig. 2.]

Cells were further cultured for seven days with or without Dox and the number of colonies was counted at day 21. To avoid overestimating the reprogramming efficiency that was caused by splitting of the culture, Dox treatment was continued for one of the culture duplicates and withdrawn from the other (Fig. 1-B). Surprisingly, most of the GFP-negative colonies from cells that were transfected with OKS+cM became GFP-positive when Dox was withdrawn and the positive rate reached to $89.83 \pm 4.98\%$ at day 21 (Fig. 2). However, most of the colonies remained GFP-negative when the Dox treatment was continued, while the number of colonies increased. The positive rate was only $4.53 \pm 0.45\%$ at day 21 (Fig. 2).

Interestingly, when the original MKOS was used as a vector, the number of colonies tended to reach a plateau at around 14 days of culture. In contrast, the colony number continued to increase at least to 21 days when OKS+cM was used (Fig. 2). In the case of OKS+cM combined with the Dox withdrawal, the numbers of

Oct3/4-GFP-positive colonies in three trials were 321, 169 and 324 at 21 days of culture and the reprogramming efficiencies were 0.32%, 0.17% and 0.32%, respectively. This efficiency ($0.27 \pm 0.05\%$) was higher than that obtained by retroviral vectors (generally 0.01-0.1%, (Hochedlinger & Plath 2009)).

2.2. Establishment of iPSC lines.

Fourteen days after the Dox addition, MEFs that were transfected with OKS+LM formed many EOS-GFP-positive colonies with mouse ESC-like morphology, while MEFs transfected with OKS+cM formed a few EOS-GFP-positive colonies, and many EOS-GFP-negative and ESC-like colonies. In the case of OKS+LM (n=3), the average numbers of EOS-GFP-positive colonies and ESC-like colonies were 28.33 ± 2.19 and 48.33 ± 6.64 respectively, and the rate of EOS-GFP-positive colonies was $59.68 \pm 4.06\%$, while in the case of OKS+cM the average numbers of EOS-GFP-positive colonies and ESC-like colonies were 25.00 ± 3.61 and 305.33 ± 17.57 respectively, and the rate of EOS-GFP-positive colonies was $8.10 \pm 0.75\%$. These results were consistent with those obtained from the section 2.1.

To confirm the similarity of *Oct3/4*-GFP reporter and EOS reporter, we reprogrammed GOF18 MEFs which were simultaneously transfected with EOS-RFP reporter. In the case of OKS+LM, we confirmed that all of EOS-RFP-positive colonies were also *Oct3/4*-GFP-positive (Fig. 3-A-C and Fig. S1-J) and most of *Oct3/4*-GFP-positive colonies were also EOS-RFP-positive (Fig. S1-J). In the case of OKS+cM, although some EOS-RFP-positive and *Oct3/4*-GFP-negative colonies were appeared at day 14 (Fig. S1-A-C), the appearance of many EOS-RFP-positive colonies after the withdrawal of Dox was the same with that of *Oct3/4*-GFP (Fig. S1-G-I). All EOS-RFP-positive colonies were also *Oct3/4*-GFP-positive 7 days after Dox withdrawal (Fig. S1-J).

Two EOS-GFP-positive colonies from OKS+cM and four EOS-GFP-positive colonies from OKS+LM were picked up (Fig. 3-D and E and Fig. S2-A and B), harvested by trypsinization and transferred to a new SNL feeder layer. Half of these colonies were derived from MEFs that were transfected with TagRFP simultaneously. After one passage, Dox was withdrawn and cells were maintained with N2B27-mESM supplemented with LIF/2i on fibronectin-coated dishes.

[Insert Fig. 3.]

[Insert Fig. 4.]

The all of resulting cell lines showed several iPSC-like cell characteristics: mouse ESC-like morphology, including round shape, large nuclei and scant cytoplasm, and strong expression of EOS-GFP (Fig. 3-F and G). These cells showed normal karyotype (Fig. 3-H, S2-G and S3-E, 2n = 40). EOS-GFP expression was low in cells from colonies with partial EOS-GFP expression (Fig. S2-A-D). However, the EOS-GFP expressing cells were readily concentrated by puromycin selection, indicating that selection by the EOS reporter was also functional (OKS+cM pu, Fig. S3-A and B). The EOS-GFP-expressing cells grew well beyond several months without serum or a feeder layer and showed strong alkaline phosphatase activity (Fig. 3-I and J, Fig. S2-E and F and S3-C and D). Immunocytological analysis also showed that the cells were positive for mouse ESC markers, such as OCT3/4, NANOG and SSEA-1 (Fig. 3-K-S, Fig. S2-H-P and Fig. S3-F-N). Semiquantitative RT-PCR analysis showed that the iPSCs expressed many undifferentiated ESC-specific genes, including *Oct3/4*, *Sox2*, *Klf4*, *Nanog*, *Rex1* (also known as *Zfp42*), *Fgf4*, *Lefty1* and *ERas* (Fig. 4-A) and did not express fibroblast-specific genes such as *Fibrillin2* (*Fbn2*) and *collagen, type V, alpha 2* (*Col5a2*) (Fig. 4-A). Furthermore, quantitative real-time RT-PCR analysis showed that

exogenous transcription factors were downregulated in the iPSCs when Dox was withdrawn from the culture medium (Fig. 4-G). Interestingly, endogenous *Oct3/4*, *Sox2* and *Klf4* were upregulated in the absence of Dox (Fig. 4-B-D), whereas endogenous *c-Myc* and *L-Myc* were downregulated in the same condition (Fig. 4-E and F).

To analyze pluripotency of the iPSC lines, we assayed cell differentiation in vitro and in vivo. First, these cells formed floating EBs, which differentiated into cells expressing GFAP (an ectoderm marker), ASM (a mesoderm marker) and AFP (an endoderm marker), indicating that the iPSCs differentiated into three germ layers (Fig. 5-A-C and Fig. S5-A-C). Second, we injected the iPSCs subcutaneously into nude mice. Four weeks after injection, these cells formed teratomas containing cells from all three germ layers, including skin epithelium structures (ectoderm), cartilage structures (mesoderm) and ciliated epithelium structures (endoderm) (Fig. 5-D-F and Fig. S5-D-F). We confirmed that all iPSC lines could form teratomas (Fig. S4). Finally, the iPSCs were injected into mouse blastocysts and the resulting embryos were transferred into five pseudopregnant mice. A RFP OKS+cM clone and a RFP OKS+LM clone that showed high EOS-GFP and RFP fluorescence were used for injections to host blastocysts. Twenty-eight live OKS+LM embryos and 24 live OKS+cM embryos were obtained from two pseudopregnant mice at E13.5. Twenty-six of the OKS+LM embryos and 5 of the OKS+cM embryos were chimeras judging by their RFP fluorescence and eye color. The OKS+LM chimeras showed markedly high chimerism (Fig. 5-G and H and Fig. S5-G and H) and the genital ridges showed high RFP fluorescence, indicating that the iPSCs differentiated to genital tissues (Fig. 5-I-K and Fig. S5-I-K). Live pups provided further evidence of chimerism as shown by their coat colors from three pseudopregnant mice (Fig. 5-L and Fig. S5-L). Finally, we confirmed the germline transmission of the OKS+LM iPSCs judged by coat color in the progenies of chimeras (Fig. 5-M, 7, 2 and 6 germline chimeras per 11, 9 and 11 newborn mice, respectively). Although germline

progeny was not obtained from OKS+cM chimeras to date, RFP-positive elongated spermatids were observed in the lumina of the seminiferous tubules of OKS+cM chimeras (Fig. S5-M-R). These data clearly showed that our established iPSC lines were authentic iPSC lines. However, we could not detect clear EOS-GFP fluorescence in the genital ridges (Fig. 5-I-K and Fig. S5-I-K), indicating that an EOS reporter might not be functional in E 13.5 primordial germ cells (PGCs).

[Insert Fig. 5.]

3. Discussion

The preceding results show that it is possible to use PB transposition of Dox-inducible reprogramming factors and an EOS reporter to establish iPSC lines with high efficiency and without the use of viral vectors.

Interestingly, in the case of OKS+cM, many *Oct3/4*-GFP-negative and ESC-like colonies appeared when exogenous factors were expressed. However, these colonies turned out to be *Oct3/4*-GFP-positive when Dox was removed from the culture and the expression of exogenous factors was turned off. Possible explanations of this phenomenon include: 1) intensive expressions of exogenous factors could suppress endogenous *Oct3/4* expression directly by negative feedback regulation (Pan *et al.* 2006), 2) residual expressions of exogenous factors in reprogrammed cells could impede the correct transcriptional network in these cells and results in suppression of endogenous *Oct3/4* indirectly (Sridharan *et al.* 2009), and 3) in the case of OKS+cM, partially reprogrammed cells could be generated more easily when exogenous factors were expressed and acquisition of full pluripotency was accelerated by the shut-off of exogenous factors.

Reprogramming by OKS+cM requires introduction of at least four vectors (OKS, cM, rtTA and PBase) into each cell, while MKOS can be transfected by three vectors (MKOS, rtTA and PBase). Therefore, we expected that MKOS would be more efficient at transfecting MEF cells than OKS+cM. However, the efficiency of reprogramming by OKS+cM was much higher than that by MKOS (Fig. 2). This indicates that OKS+cM is more effective at reprogramming than MKOS and that the expression levels of each reprogramming factor might be better with OKS+cM. It should be mentioned that OKS+LM was much more efficient at reprogramming than OKS_{LM} (Fig. 2), which emphasizes the importance of the levels of each reprogramming factor. However, increasing the number of insertions of PB vectors into a genome makes it

difficult to remove these vectors by reexpression of PBase (Woltjen *et al.* 2009). Two strategies have been proposed to eliminate inserted PB vectors efficiently. One is negative selection of vector-free cells by herpes virus thymidine kinase (HSVtk) -fialuridine (FIAU) selection system (Yusa *et al.* 2009) and the other is reducing the number of vectors (Woltjen *et al.* 2009). To reduce the number of vectors, further studies are needed to determine the optimal levels of each reprogramming factor and the optimal order of the four reprogramming factors in a single polycistronic vector.

The iPSCs used for chimera production in this experiment have a hygromycin-resistance gene behind the TagRFP gene (Fig. 1-A). Therefore, differentiated cells that are derived from the iPSCs can be recovered from the chimera by hygromycin selection. The selected cells already have the Dox-inducible factors and can be used to induce reprogramming by only the addition of Dox. The “secondary” reprogramming system is useful for analyze reprogramming under genetically identical conditions (Brambrink *et al.* 2008; Stadtfeld *et al.* 2008).

Here, we showed that only one transfection of multiple plasmids containing the minimal and essential reporter and reprogramming factors is sufficient for production of iPSCs. We constructed a PB vector containing an EOS reporter that was originally used in the lentiviral vector system (Hotta *et al.* 2009). We confirmed in the present experiment that the PB-EOS vector was functional in generation of mouse iPSCs and that additional reporters and selection markers were not necessary. EOS reporter contains short gene regulatory regions that are conserved in various mammalian species (Hotta *et al.* 2009). EOS may also be used as a universal pluripotent reporter in various mammalian species. Therefore, the system could be used to generate iPSCs in various mammalian species. Indeed, we have observed EOS-reporter expression during iPSC generation in pig, dog, rabbit and cattle (data not shown). In lentiviral system, however, it is essential to use special instruments for biological containment and it

1 requires complicated procedures. While, PB system does not require high containment
2 level and it requires only one transfection. Because the facility for generation of
3 transgenic large animals that has high containment level is extensive and expensive,
4 PB system may be useful for generation of transgenic large animals.

5 In large domestic animals, some iPSC lines have been established but all of
6 them maintain their pluripotency under the support of exogenous factors (Esteban *et al.*
7 2009; Ezashi *et al.* 2009; Shimada *et al.* 2009; Wu *et al.* 2009). It is necessary to
8 determine an optimal culture condition for the establishment and maintenance of
9 transgene-independent iPSCs. In previous study, Yusa *et al.* (Yusa *et al.* 2009) also
10 successfully reprogram mouse somatic cells with high efficiency by PB system. However,
11 since they used a constitutive CAG promoter to drive the reprogramming factors, it is
12 difficult to regulate transgene expressions. In contrast, our system can easily regulate
13 these expressions and enables us to analyze whether reprogrammed cells can maintain
14 pluripotency without the expression of exogenous transcription factors. Therefore, our
15 system can be applied to screen an optimal culture condition for generating authentic
16 iPSCs in various mammalian species.

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4. Experimental procedures

4.1. Vector construction

To prepare the OKS PB vector (PB-TET-OKS, Fig. 1-A), pCX-OKS-2A was digested by EcoRI, blunted and introduced into blunt-ended PB-TET-MKOS at the BsrGI site. To prepare the OKS_{LM} PB vector (PB-TET-OKS_{LM}, Fig. 1-A), PB-TET-OKS was amplified by PCR using a reverse primer eliminating the Sox2 stop codon, and L-Myc containing a 2A sequence in the upstream was amplified by PCR using a forward primer containing a 2A sequence (Table S1). Then, the ligation of these DNA fragments was performed. To prepare the L-Myc PB vector (pPB-TET-L-Myc, Fig. 1-A), the protein coding regions of these genes were amplified by PCR using oligonucleotide primers as described in Table S1. The amplified DNA fragments were digested by XhoI and NotI and introduced into pPB-hCMV1-cHApA at the XhoI and NotI sites. To prepare the EOS PB vector (pPB-EOS-C(3+)-EiP, Fig. 1-A), PL-SIN-EOS-C(3+)-EiP were digested by BamHI and ClaI, blunted and introduced into the blunt-ended empty PB vector. To prepare the TagRFP PB vector (pPBCAG-TagRFP-IH, Fig. 1-A), pTagRFP-N were digested by XhoI and NotI and introduced into pPBCAG-cHA-IH at the XhoI and NotI sites. To prepare the EOS-RFP PB vector, pPBCAG-cHA-IP were digested by BglII and BamHI and introduced into pPB-EOS-C(3+)-EiP vector at the BglII and Bam HI site. pTagRFP-N were digested by BglII and NotI and introduced into pPBCAG-cHA-IP at the BglII and NotI sites. Then, two resulting vectors were digested by BglII and ClaI and ligated.

The original vectors (PB-TET-MKOS, PB-CAG-rtTA Adv, pCX-OKS-2A and PL-SIN-EOS-C(3+)-EiP) were obtained from Addgene (plasmid 20959, plasmid 20910, plasmid 19771 and plasmid 21313) (Okita *et al.* 2008; Hotta *et al.* 2009; Woltjen *et al.* 2009). The empty PB vector, the c-Myc PB vector (pPB-TET-c-Myc), pPBCAG-cHA-IH and pPBCAG-cHA-IP were kindly gifted from Dr. Hitoshi Niwa at RIKEN Center for

Developmental Biology. The PBase vector (pCAG-PBase) was kindly gifted from Dr. Austin Smith at University of Cambridge (Guo *et al.* 2009).

4.2. Preparation of MEFs

For preparation of MEFs, embryos were collected at embryonic day (E) 13.5-15.5. After removal of the heads and visceral tissues, the remaining bodies were washed in fresh PBS, minced and the isolated cells were maintained in Dulbecco's modified eagle medium (DMEM, Invitrogen) containing 10% fetal bovine serum (FBS, JRH Biosciences), penicillin (Sigma) and streptomycin (Sigma).

4.3. Analysis of reprogramming efficiency

To examine the effect of vector constructions on the efficiency of iPSC generation, MEFs that originally having *Oct3/4*-GFP reporter (GOF18 MEFs) were used. GOF18 MEFs were plated at 1×10^5 cells per 35 mm dish and incubated overnight. Cells were then transfected using 16 μ l of Lipofectamine LTX transfection reagent (Invitrogen) and 2 μ l of Plus reagent (Invitrogen) mixed with 1 μ g of PBase vectors (pCAG-PBase) and 1 μ g of reprogramming PB vectors (PB-TET-MKOS, PB-TET-OKS, the combination of PB-TET-OKS and pPB-TET-c-Myc (OKS+cM), the combination of PB-TET-OKS and pPB-TET-L-Myc (OKS+LM) or PB-TET-OKS_{LM}) and rtTA PB vectors (PB-CAG-rtTA Adv).

The transfected cells were cultured in 10% FBS DMEM supplemented with Primocin (InvivoGen). One day after transfection, the medium was replaced by Glasgow minimum essential medium (GMEM, Sigma) containing 10% FBS, 2 mM L-glutamine (MP Biomedicals), 1 mM sodium pyruvate (Sigma), $1 \times$ MEM non-essential amino acids (NEAA, Invitrogen), 0.1 mM 2-mercaptoethanol (2-ME, Wako), penicillin and streptomycin (10% FBS GMEM ESM) supplemented with 1.5 μ g/ml doxycycline (Dox,

Sigma). Seven days after the Dox addition, the medium was replaced by GMEM containing 15% Knockout Serum Replacement (KSR, Invitrogen), 0.3% FBS, 2 mM L-glutamine, 1 mM sodium pyruvate, $1 \times$ NEAA, 0.1 mM 2-ME, penicillin and streptomycin (mouse ESC medium, mESM) supplemented with leukemia inhibitory factor (LIF). As a source of LIF, we used the commercially available LIF (ESGRO (1000 U/ml), Millipore) or LIF-conditioned medium (1:1000 dilution) from COS-7 or 293FT cell cultures that had been transduced with a mouse LIF-encoding vector (Niwa *et al.* 1991; Yoshida-Koide *et al.* 2004). The medium was changed every day.

The number of ESC-like colonies and the expression of *Oct3/4*-GFP were compared 14 days after the Dox addition (d14) (Fig. 1-B). In this experiment, to measure reprogramming efficiency precisely, these cells were not passaged during reprogramming procedure because reprogramming efficiency was overestimated by splitting of culture (Smith *et al.* 2010).

4.4. Establishment of iPSC lines using the EOS reporter

Reprogramming of GOF18 MEFs with EOS-RFP reporter were performed according to 4.3. section.

To efficiently establish mouse iPSC lines from MEFs that did not have reporter genes, 1×10^5 cells of 129B6F1 MEFs were transfected with OKS+cM or OKS+LM and simultaneously with PB-CAG-rtTA Adv, pCAG-PBase and the EOS PB vector (pPB-EOS-C(3+)-EiP). For the analysis of teratoma formation and chimera production, a part of cells were also simultaneously transfected with a constitutive TagRFP expressing PB vector (pPBCAG-TagRFP-IH).

One day after transfection, Dox was supplemented into the culture. Four days after the Dox addition, 1×10^5 cells of cells (approximately 1/8 of dish) were reseeded on the SNL feeder layer and the medium was replaced by mESM supplemented with LIF

at the next day (Fig. 1-C). Fourteen days after the Dox addition, primary colonies were picked up and chemically dissociated and transferred on the SNL feeder layer in 48-well plates. Following the passage, Dox was withdrawn and the established iPSCs were maintained with 50% Neurobasal medium (Invitrogen) and 50% DMEM/F12 (Invitrogen) containing $0.5 \times \text{N2}$ (Invitrogen), $0.5 \times \text{B27}$ (Invitrogen), $1 \times \text{L-glutamine}$, penicillin and streptomycin (Invitrogen), 0.05% BSA (Sigma) and 0.15 mM 1-thioglycerol (Sigma) (N2B27-mESM) supplemented with LIF, 1 μM PD0325901 and 3 μM CHIR99021 (2i, Stemgent) on human plasma fibronectin (Millipore) coated dishes. The medium was changed every day.

4.5. Alkaline phosphatase staining and immunohistochemical staining

Alkaline phosphatase staining was performed using the Vector Alkaline Phosphatase Substrate kit (Vector) according to the manufacturer's instructions.

For immunofluorescence analysis, cells were fixed with PBS containing 3.7% paraformaldehyde for 10 min at room temperature. After washing with PBS, cells were blocked with 10% goat serum and 0.1% Triton X-100 for 45 min at room temperature and were treated with primary antibodies overnight at 4°C. Primary antibodies included Oct3/4 (1:25, SC-5279, Santa Cruz), Nanog (1:250, AB5731, Millipore), SSEA-1 (1:25, MC-480 (SSEA-1)-s, Developmental Studies Hybridoma Bank), glial fibrillary acidic protein (GFAP, 1:100, Z0334, DAKO), actin smooth muscle (ASM, 1:1000, MS-113-P0, Thermo) and α -fetoprotein (AFP, 1:100, MAB1368, R&D Systems). Alexa Fluor 594 goat anti-mouse IgG or IgM (1:500, Molecular Probes) and Alexa Fluor 594 goat anti-rabbit IgG (1:500, Molecular Probes) were used as secondary antibodies. Nuclei were stained with 1 $\mu\text{g/ml}$ Hoechst 33342 (Sigma).

4.6. Semiquantitative RT-PCR and quantitative real-time RT-PCR

Total RNAs of cells were prepared using a RNeasy Mini Kit (QIAGEN). DNase (TURBO DNA-free Kit, Ambion) was added to preparations to avoid genomic contamination. For reverse transcription, ReverTra Ace (Toyobo) and Random Primer (Invitrogen) were used, according to the manufacturer's instructions. PCR was performed with ExTaq (Takara). For real-time PCR, Power SYBR Green PCR Master Mix (Applied Biosystems) was used. Transcription levels were determined in triplicate reactions and normalized to Gapdh. Primer sequences are shown in Table S1.

4.7. Differentiation of iPSCs in vitro

To form embryoid bodies (EBs), established iPSCs were harvested by trypsinization and transferred to MPC-treated round-bottom dishes (Nunc) in Iscove's modified Dulbecco's medium (IMDM, Invitrogen) containing 15% FBS, 2 mM L-glutamine, 1mM sodium pyruvate, $1 \times$ NEAA, 0.1 mM 2-ME, penicillin and streptomycin. The medium was changed every other day. After 3 days of culture, the floating EBs were transferred to gelatin-coated plates and cultured in the same medium for another 3 days. Immunohistochemistry was analyzed as described in 2.5. section.

4.8. Teratoma formation

iPSCs were harvested by trypsinization, transferred into tubes and centrifuged, and the resulting cell pellets were suspended in 10% FBS DMEM. 1×10^6 cells were injected subcutaneously into the dorsal flank of nude mice (BALB/c nude (nu/nu), Oriental BioService). Four weeks after injection, tumors were dissected, weighted, and fixed with PBS containing 3.7% paraformaldehyde. Paraffin-embedded tissues were sliced and stained with hematoxylin and eosin.

4.9. Chimera production

1 To generate chimeric mice, iPSCs were injected into embryos (CD1, Oriental
2 BioService) at the blastocyst stage. The blastocysts were transferred into the uterine
3 horns of five pseudopregnant mice (CD1 background). Chimerism of live embryos and
4 pups were judged by RFP fluorescence, eye color and coat color. After mating with
5 C57BL/6 mice, germline transmission of iPSCs was judged by coat color of progeny. The
6 contribution of RFP-positive cells into the lumina of seminiferous tubules was analyzed
7 by confocal fluorescence microscopy.

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7. Figure Legends

Fig. 1. Reprogramming of mouse somatic cells and establishment of iPSCs using PB vectors. (A) Construction of PB vectors used in this study. (B) Timeline for the analysis of reprogramming efficiency of cells. (C) Timeline for the establishment of iPSC lines.

Fig. 2. Reprogramming efficiency of MEFs by various PB vectors. (A) The numbers of ESC-like and *Oct3/4*-GFP-positive colonies at days 14 and 21 in the presence or absence of Dox. (B) The average numbers of ESC-like and *Oct3/4*-GFP-positive colonies at days 14 and 21 in the presence or absence of Dox. Open bars show the number of total ESC-like colonies and closed bars show the number of *Oct3/4*-GFP-positive colonies. +, Dox was withdrawn at day 14; –, Dox was supplemented for the whole term.

Fig. 3. Characterization of established iPSC lines. The morphology of a EOS-RFP and *Oct3/4*-GFP-positive colony (A, RFP-positive image; B, GFP-positive image; C, phase contrast image) derived from cells that were transfected with OKS+LM. The morphology of a EOS-GFP-positive colony (D, GFP-positive image; E, phase contrast image) derived from cells that were transfected with OKS+LM. The established iPSC line (F, GFP-positive image; G, phase contrast image) transfected with OKS+LM. Karyotyping of OKS+LM-induced iPSCs (H). Alkaline phosphatase activity in OKS+LM-induced iPSCs (I, activity in a culture dish; J, activity in colonies). OCT3/4 (K, OCT3/4 staining; L, EOS-GFP-positive image; M, Hoechst staining), NANOG (N, NANOG staining; O, EOS-GFP-positive image; P, Hoechst staining) and SSEA-1 (Q, SSEA-1 staining; R, EOS-GFP-positive image; S, Hoechst staining) expression in OKS+LM-induced iPSCs.

Fig. 4. Endogenous and exogenous gene expression specific for undifferentiated ESCs in

iPSCs. To distinguish the amplification from genomic contamination, primers for the expression of *Nanog*, *Rex1*, *Fgf4*, *ERas*, *Fbn2* and *Col5a2* were designed to span intron-exon boundaries. A OKS+cM-induced iPSC line after puromycin selection was denoted by pu. iPSC lines simultaneously transfected with a TagRFP were denoted by RFP. Quantitative real-time RT-PCR analysis for endogenous reprogramming factors and transgens (B-G). Relative gene expressions of endogenous reprogramming factors (B, *Oct3/4*; C, *Sox2*; D, *Klf4*; E, *c-Myc*; F, *L-Myc*) to ESCs. Relative transgene expressions in iPSCs (G) to the cells that were cultured continually in the presence of Dox. Error bars indicate SE between three technical replicates. * $P < 0.05$, Student's t-test.

Fig. 5. Pluripotency of established iPSC lines. Immuno-histochemical staining by three-germ-layer markers in differentiated cells derived from OKS+LM-induced iPSCs. Glial fibrillary acidic protein (A, ectoderm), actin smooth muscle (B, mesoderm) and α -fetoprotein (C, endoderm) were used for the markers. The derivative of three germ layers (D, skin epithelium structures; E, cartilage structures; F, ciliated epithelium structures) in teratomas derived from OKS+LM-induced iPSCs. Contribution of OKS+LM-induced iPSCs to mouse embryonic development (G, Bright-field image; H, RFP image). Embryos were analyzed by fluorescence microscope at E 13.5. Contribution of OKS+LM-induced iPS cells to genital ridges (I, Bright-field image; J, GFP image; K, RFP image) at E13.5. Live chimeric mice (L) derived from OKS+LM-induced iPSCs. Germline-transmitted F1 progenies (M) derived from OKS+LM-induced iPSCs.

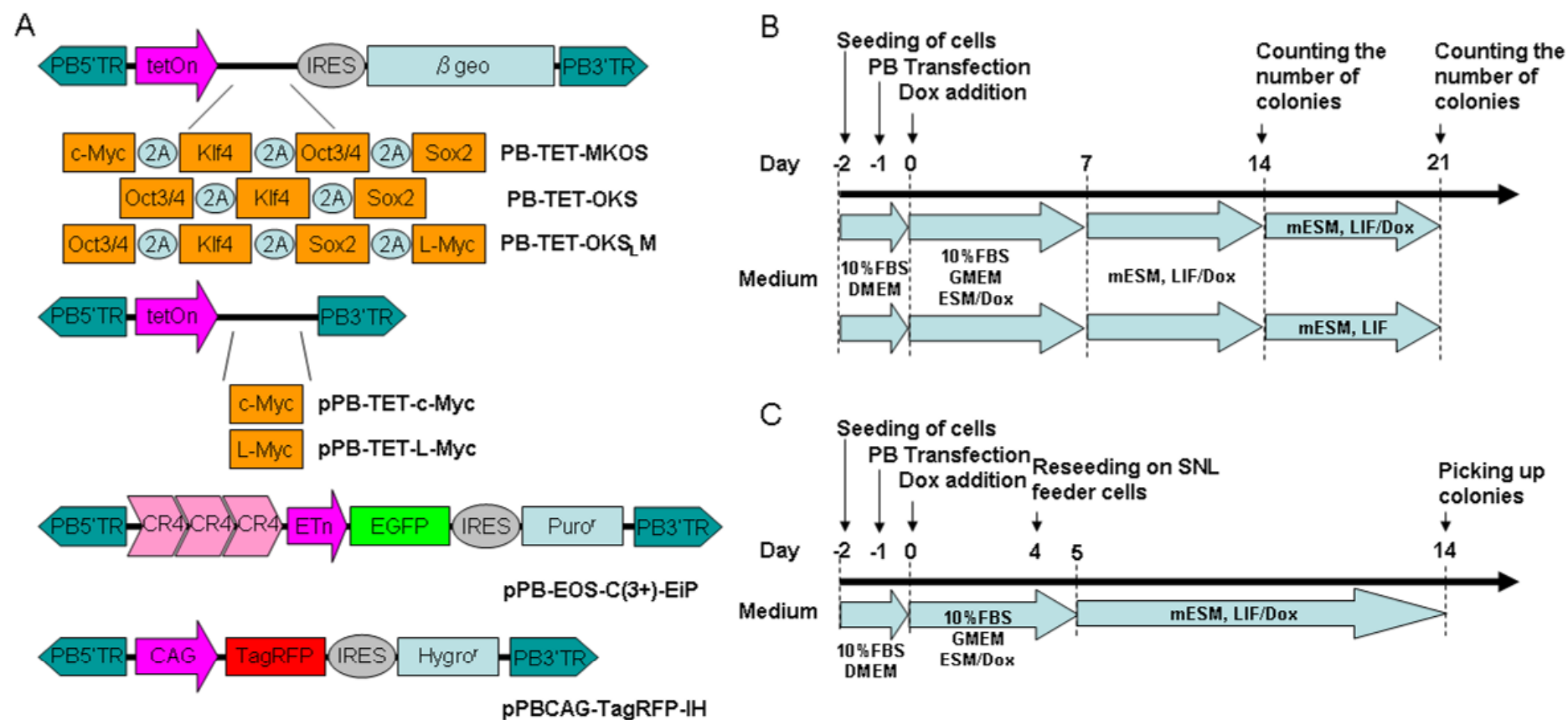


Fig. 1

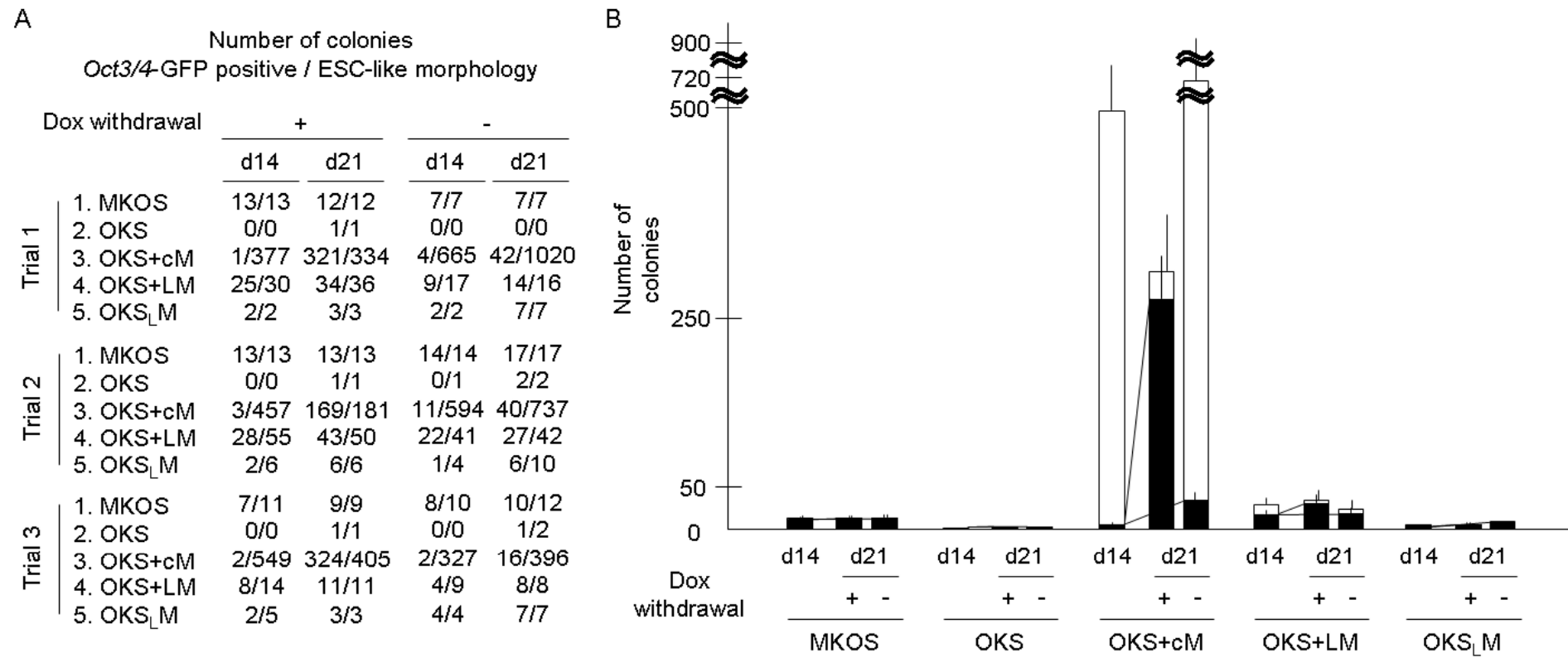


Fig. 2

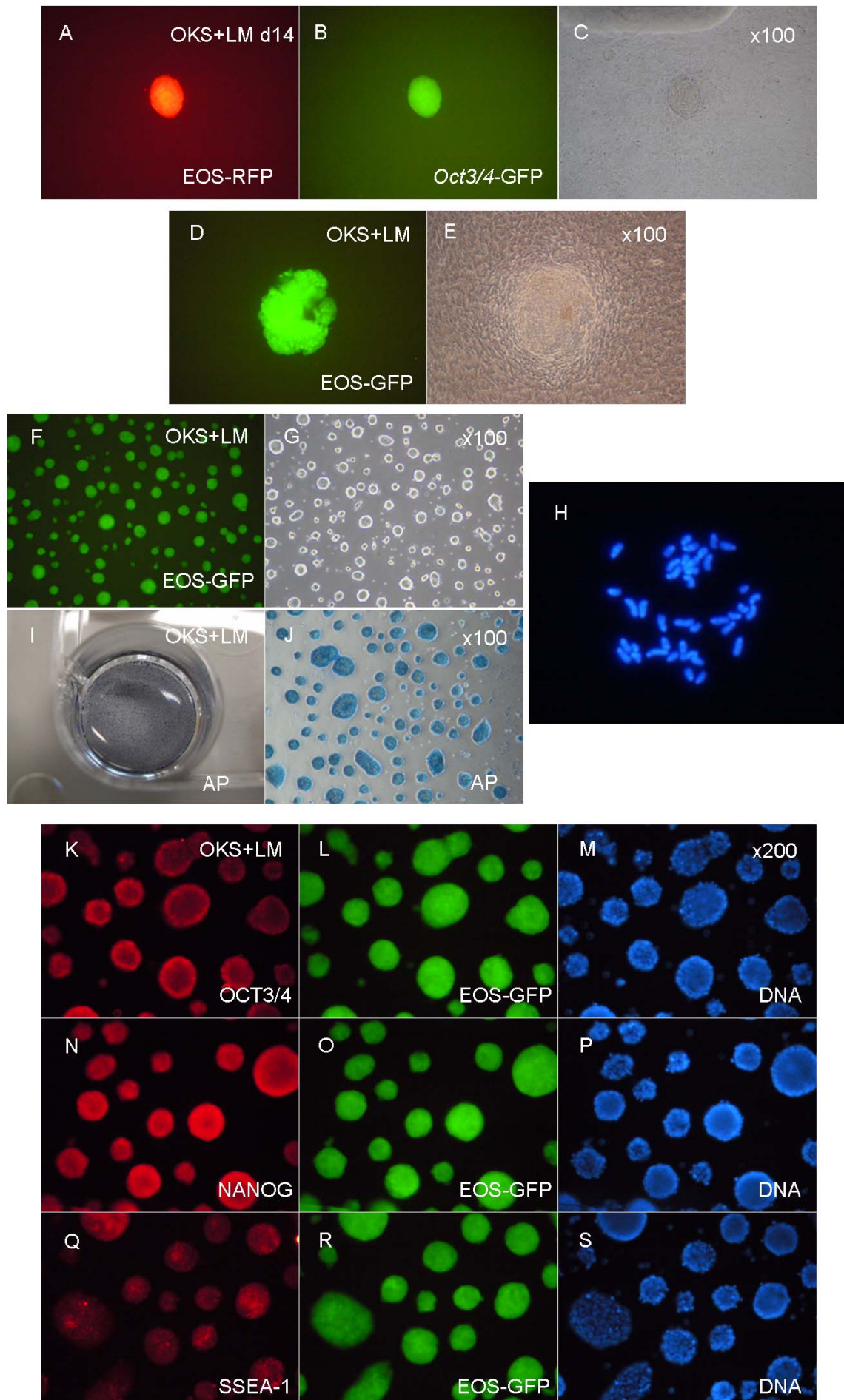


Fig. 3

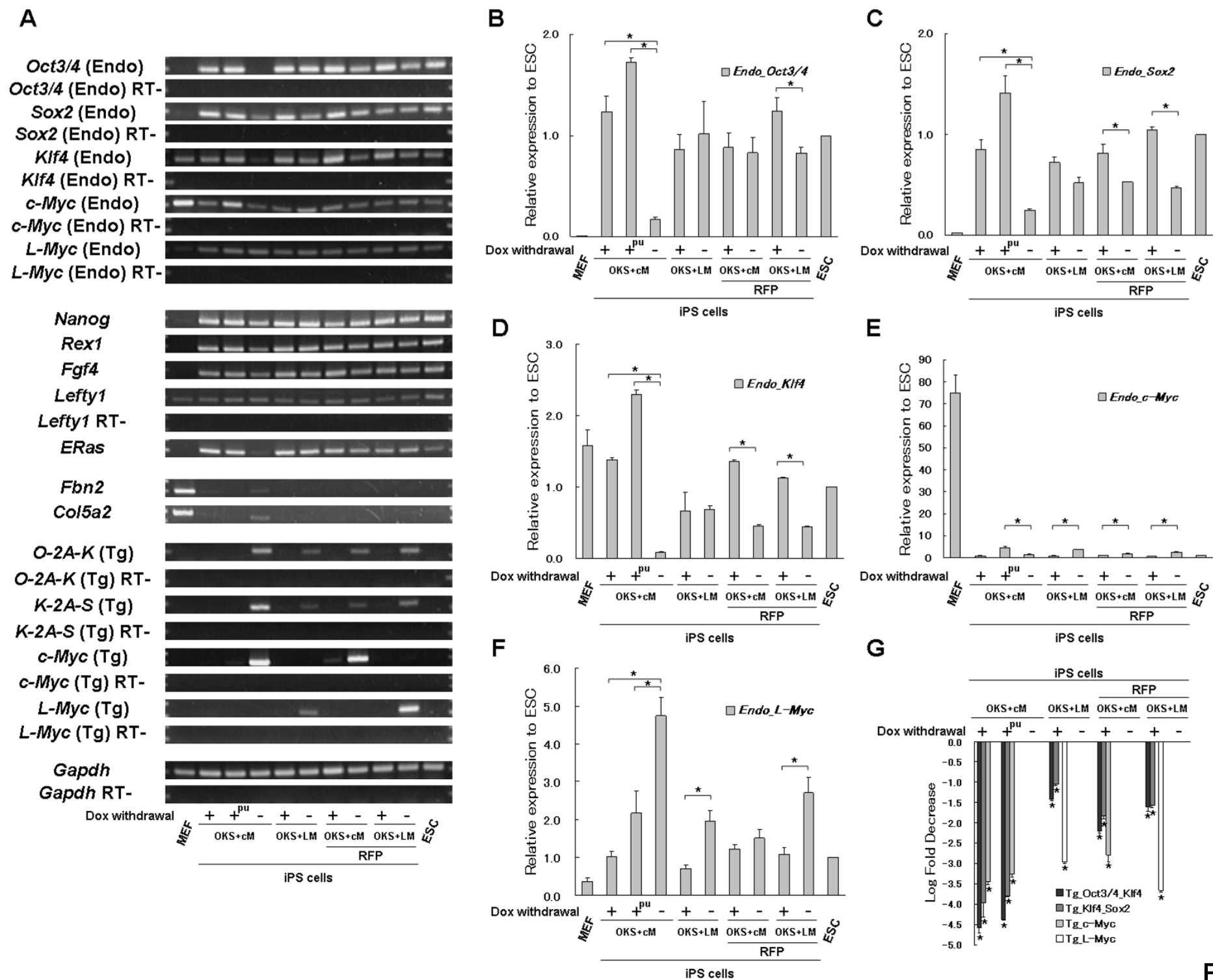


Fig. 4

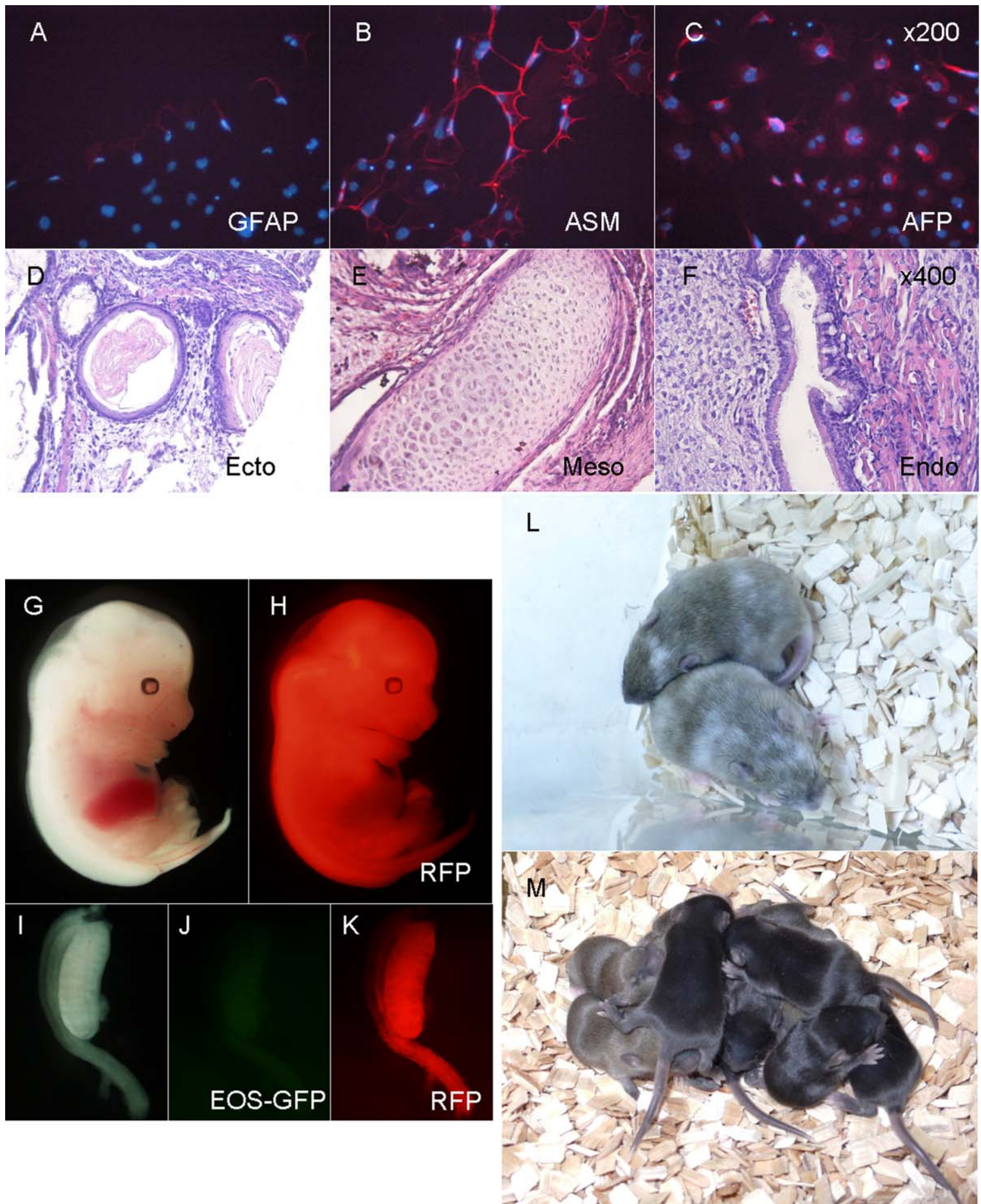


Fig. 5